Comments on NTP Co metal study (NTP TR 581 draft, 2013)

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General comments

The current NTP study investigated the inhalation hazard of Co metal. As an overarching comment, it needs to be considered that Co is an essential metal to microorganisms, such as ruminant bacteria, which incorporate it into cobalamin (vitamin B12). Vitamin B12, in turn, is essential to humans, its main roles being in blood cell formation, DNA synthesis and energy metabolism. Effects related to tissue levels of Co are therefore expected to follow a U-shaped dose-response, with adverse effects in the deficiency as well as in excess range.

Inhalation toxicity of Co comprises chemical effects as well as local particle effects in the respiratory tract, not unlike many other dusts. Both physical (local) as well as chemical considerations are important when interpreting the various biological data in this report.

The interpretation of the local carcinogenic effect of Co needs to consider the lack of mutagenicity, which has been confirmed by recent studies, many of them mentioned in the current NTP report. A genotoxicity database for Co has also been established by the Cobalt Development Institute (CDI), further corroborating the negative findings. This database will undergo external peer-review and will be publicly available in early 2014.

In summary, the findings of the current NTP study together with the lack of direct mutagenicity of Co suggest a threshold mechanism for Co in both systemic as well as local effects.

Overall study design and outcome

The exposure levels of 1.25, 2.5 and 5.0 mg/m^3 for the 2-yr studies were based on the results from 14 and 90-day pre-chronic studies. The primary findings in the pre-chronic studies of Co that drove the selection of exposure levels for the chronic study were decreases in body weight gain relative to the unexposed control groups.

There may be indications that Co exposure levels for the chronic study were relatively high in terms of overall toxicity (e.g., reduced survival and early reduced bodyweights in female rats, greater than 10% reduced bodyweights, exposure related “thinness”). Also, in

comparison to the previous 2-year inhalation studies by NTP (cobalt sulfate) the exposure levels were relatively high: The highest exposure concentration in the previous Co sulfate study (3 mg Co sulfate heptahydrate) is equivalent to less than half of the lowest exposure concentration of cobalt metal (1.25 mg/m³) delivered to the animals in the current study. (The exposure concentrations of the two NTP Co inhalation studies are further examined in section “Comments on report text” of this document).

The study is of high quality with excellent data reporting. The study design (particle size distribution, purity of test item, test species, exposure duration) was suitable to examine the inhalation hazard of Co metal. In addition, valuable and original data on lung clearance were generated.

Chronic study: local effects (respiratory tract)

Non-neoplastic findings

Various types of non-neoplastic lesions were found in the lungs of male and female rats. The incidences of alveolar epithelium hyperplasia, alveolar proteinosis, chronic active inflammation, and bronchial epithelium hyperplasia in all exposed groups were significantly greater than those in the control groups. The severities of some lesions increased with increasing exposure concentrations, others however were maximal at the lowest exposure dose already (e.g., alveolar proteinosis and alveolar hyperplasia in male rats). This spectrum of non-neoplastic lesions invariably occurred together and presented as a complex mix.

A spectrum of non-neoplastic lesions occurred in the nose of exposed male and female rats including chronic active and suppurative inflammation, changes of the olfactory epithelium (respiratory metaplasia, atrophy, hyperplasia, hyperplasia of basal cells, necrosis), changes of the respiratory epithelium (hyperplasia, squamous metaplasia, necrosis), and turbinate atrophy.

Local non-neoplastic findings in male and female mice were similar.

Neoplastic findings

Significantly increased rates of tumors (alveolar/bronchiolar carcinoma) were seen in the lung of all rats (males and females) exposed to 1.25, 2.5, or 5 mg/m³ as compared with those in the chamber controls (males: 32%, 68%, 72% vs. 0%, females: 18%, 34%, 60% vs. 0%).

The findings in mice were similar.

Considering the concentration-response relationship with positive trends of tumor formation in the lung of both sexes of rats and mice and taking into account only the concurrent study controls the increased number of alveolar/bronchiolar carcinoma in both species at the lowest concentration (1.25 mg/m³) can be considered treatment-related.

The myriad of tumors observed suggests both a chemical and particulate mode of action. Bronchoalveolar adenomas and carcinomas are often found with both particulate and non-particulate carcinogens, while “cystic keratinizing epitheliomas” only occur in the presence of physical particulates, often of an inert quality.

The incidences of the various types of non-neoplastic lesions, which were significantly increased in all exposed groups of both species of each sex, indicates that the lowest exposure concentration of 1.25 mg/m³ represents a LOAEC for non-neoplastic effects on the lower respiratory tract and also for pulmonary carcinogenicity. A NOAEC for local (non-neoplastic) effects on the respiratory tract and for pulmonary tumor formation could not be derived from these studies.

The comparison of the exposure-response for non-neoplastic and neoplastic lesions supports the notion that inflammation is a very highly sensitive response to Co metal inhalation both in terms of timing (acute, immediate), as well as exposure level. It appeared that inflammation preceded cancer both in terms of “dose” as well as timing.

It needs to be stressed that all derived OELs for humans are aimed at preventing the earliest and most sensitive sign of Co effects in the lung, namely inflammation, covering lifetime exposure. This effect can be assessed qualitatively by self-report of coughing and wheezing, or by quantifying lung function parameters such as FEV1 (forced expiratory volume during 1 second).

Induction of oxidative stress by cobalt

Cobalt metal and cobalt(II) ions have been shown to generate different reactive oxygen species (“ROS”; superoxide anion radical, hydrogen peroxide, hydroxyl radical, or singlet oxygen). Of those ROS, mainly hydroxyl radical and singlet oxygen can lead to oxidative DNA damage, as was also observed in the current NTP Study.

The above observations lend support to the hypothesis that the carcinogenic mode of action of cobalt involves sustained local inflammation (lung) and oxidative stress, without direct mutagenicity. This is further supported by genotoxicity studies of Co soluble salts and Co metal. 14 different Co substances have been tested in GLP- and OECD compliant studies and were found to be negative for mutagenicity in several *in vitro* test systems (incl. Ames tests), as well as negative for *in vivo* clastogenicity. The studies were performed by the CDI, and will be published after an external peer review (expected early 2014).

Systemic effects

Co, from inhalation of Co metal fine powder, became available systemically, as evidenced by increased Co levels in all examined tissues.

Few data are available on the bioavailability of Co via inhalation. To estimate and compare bioavailability via inhalation of different Co compounds, the CDI has generated quantitative bioaccessibility data for 28 different commercially available cobalt compounds exposed to different synthetic lung fluids. The generated results form a unique set of data that aim to improve the current knowledge on release/dissolution properties of such particles.

Four measures were used to evaluate the bioaccessibility of cobalt species if inhaled, using synthetic equivalents of alveolar fluid, interstitial fluid, lysosomal fluid, and human serum. In these fluids, the selected cobalt species showed variations in bioaccessibility. Cobalt carboxylates, cobalt metal powder, cobalt oxide and cobalt carbonate appear to be appreciably less soluble in neutral lung fluid equivalents than the lysosomal surrogate. Based on the *in vitro* data, inhaled particles of these materials would have to be phagocytised before full solubilisation would occur. This is also concluded in the NTP report (page 145), based on the current *in vivo* data.

Adrenal gland – There were exposure-concentration dependent increases in the incidences of benign and malignant pheochromocytoma (combined) in all substance-exposed male and female rats. This effect was not observed in mice.

A previous survey of NTP study data showed the induction of hyperplasia and formation of benign or malignant pheochromocytoma in the adrenal medulla of experimental animals by a number of chemicals (Ozaki et al., 2002; Greim et al., 2009). A possible correlation between marked hypoxic conditions in the lungs (lung tumours, inflammation, and fibrosis) and occurrence of pheochromocytoma has been suggested previously. Also, there is no indication for an involvement of genotoxic mechanisms in the induction of pheochromocytoma by chemicals in animals (Ozaki et al., 2002; Greim et al., 2009).

Reviews of all publicly available Co toxicokinetic studies (mainly from oral exposure) by the CDI as well as by Finley et al, 2012, do not indicate that the adrenal gland is a target organ of Co. Co levels in that tissue were not examined in the current NTP Study. In response to the NTP findings, a planned toxicokinetic study (CDI) in rats will include the adrenal gland, in order to corroborate the conclusion that the findings of pheochromocytoma are not related directly to cobalt, but are a result of hypoxic conditions in the lung.

Pancreas –There was a small increase in Islet-cell tumors in the mid- and high-dose male but not in female rats. Mice seemed to be less sensitive for this effect.

These tumors are rare and they were seen for the first time in NTP studies. They were also not observed in other studies with cobalt substances as described in this report. In this context it needs to be considered that the historical control data for this strain and exposure route in this institution are weak. The exact mechanism for induction of these tumors is not well understood and they may result from chronic inflammation of the

endocrine hormone producing tissue of the pancreas. However, no evidence of pancreatic inflammation was seen in the other studies with cobalt. Therefore, these findings should be interpreted with caution. The statement “equivocal evidence of carcinogenic activity” is considered justified.

The above-mentioned reviews of Co toxicokinetic studies (CDI and Finley et al, 2012) also provide no indication that the pancreas is a target organ of Co toxicity. Co levels in the pancreas were not examined in the current NTP Study. In response to the NTP findings, a planned toxicokinetic study (CDI) in rats will include pancreas, in order to corroborate the conclusion that this may be a spurious or equivocal finding.

Mononuclear cell leukemia (MNCL) - While there was an increase in MNCL at all exposure levels in female rats, the increase was not exposure level-related (incidence at the lowest exposure was highest). In addition, there was no significant increase in male rats.

Extremely elevated incidences of MNCL have been previously observed in a number of chronic bioassays and 2-year carcinogenicity studies in F344 rats (Haseman et al., 1998; Caldwell, 1999). The analysis of the spontaneous neoplasm incidences in F344 rats from chamber controls of 18 two-year inhalation studies carried out by the NTP revealed a frequent occurrence of MNCL in males (57.5%, range 34-70%) and in females (37.3%, range 24-54%) (Haseman et al.,1998). The data show that MNCL occurs in untreated aged rats at extremely high and variable rates. MNCL is uncommon in most other rat strains, and its background incidence has increased significantly over time (Caldwell, 1999). MNCL has not been found in other mammalian species and no histologically comparable tumor is found in humans. In the light of the well-known occurrence of MNCL in the Fisher rat, the relevance of this result for human cancer risk is questionable.

Kidney, adenoma/carcinoma combined – There was a minimal increase in the incidence of these tumors in male rats, although not statistically significant. Because of this slight increase an extended review using “step-sections” was conducted. Using these extended data there is essentially no evidence of a carcinogenic response in male rats, which is supported by no increase in tubular hyperplastic changes or in kidney tumors in female rats or in male and female mice.

The neoplasms in the kidney were slightly above the historical control data, but not statistically significant and no overall positive trend was established. They were seen only in one sex (males) and not in females and mice, were however regarded as may be exposure related. Also in this context the weakness of the historical control data need to be taken in consideration.

Co levels in the kidney were not examined in the current NTP Study. The kidney is involved in excretion of soluble Co compounds during high exposure and, in response to

the NTP findings, a planned toxicokinetic study (CDI) in rats will include kidney Co levels, in order to corroborate the conclusion that this may be a spurious or equivocal finding.

Systemic effects – general - It is interesting to note that the liver had highest Co levels, in some cases even exceeding lung Co levels, yet there were no neoplasms, nor any other significant findings, in the liver. This is a strong indication that Co is not mutagenic, and that the local cancer (lung) is caused by local persistent inflammation. It is also a strong indication that the adrenal, pancreatic and other findings are likely spurious.

Systemic findings at higher exposure levels (pre-chronic studies)

Some systemic findings occurred in the pre-chronic studies at the higher exposures (above 5 mg Co/m^3) not used in the 2-year study due to general toxicity (reduction in bodyweight).

These findings included changes in the testes of male rats and mice. The testes are a known target organ of Co toxicity, and soluble Co salts are classified as reprotoxicants Cat 1B under EU CLP. Co metal has an interim classification as Cat 2. An extensive testing program under ECHA (European Chemicals Agency) is currently ongoing to derive appropriate reprotoxicity classifications for all Co compounds.

Another systemic effect seen in the pre-chronic studies was erythrocytosis. This is another well-described target organ and effect Co, e.g. Co salts were used in 1940s and 1950s as effective medication against anemia.

Systemic findings – relevance to humans

It is important to note that systemic effects are generally not achieved by inhalation exposure in humans, due to the strong local effects in the lung. The occurrence of inflammation is prevented by occupational exposure limits, and these levels are significantly lower than the exposures utilized in the current NTP study. As was discussed earlier in these comments, the aim in protecting workers and setting human OELs is to prevent the earliest and most sensitive sign of Co inhalation, which is inflammation.

Systemic effects can probably only be achieved by targeting Co aerosol to the respiratory tract as is done in the study design of bioassays to investigate the inhalation hazard of chemicals. This type of a mono-disperse particle size distribution (PSD) with little variation (small particle size with small GSD) does not occur in the workplace. The CDI is currently investigating PSDs typical for the workplace to document the PSDs encountered by workers in some detail.

Conclusion

The finding that there is clear evidence for local carcinogenicity in the respiratory tract of rats and mice upon inhalation exposure to Co metal fine powder is supported by the data.

Comments on report text

Pertaining to page 23, section “production and use”, some additional uses for cobalt are worth mentioning: Cobalt is used in rechargeable batteries, particularly for portable devices, and is essential in the biotech industry for diagnostics. Prosthetic alloys are also an important use as well as adhesives in radial tyres, driers in paint/ink, digital storage, electronic connectors and humidity detectors in sensitive electronics (military applications) (Reference: CDI).

In reference to page 24, second paragraph, it is worth noting that cobalt was identified as critical to USA industry in the U.S. Department of Energy – Critical Materials Strategy of 2010.

The following section “Environmental and Human exposure”: “Occupational exposure to cobalt occurring during the production of cobalt powder is a major concern due to the occurrence of hard metal disease and is primarily via inhalation of dust, fumes, or mists containing cobalt, targeting the skin and respiratory tract, during the production, processing and use of hard metal”. Hard metal disease is a result of combined exposure (synergistic effect) of Co and WC during production and use of hard metal, not cobalt alone. Also, the reference to skin exposure is not relevant in context of hard metal disease.

Suggested rewording: “Occupational exposure to cobalt, occurring during the production of cobalt powder, is a major concern as it targets the skin (sensitization) and respiratory tract. Occupational exposure to cobalt in combination with WC occurs during the production, processing and use of hard metal. This is a major concern due to the occurrence of hard metal disease via inhalation of dust, fumes, or mists containing cobalt and WC.”

p. 138, 2nd para

Stabilization of HIF-1α

Up to now the exact biochemical mechanism of HIF-1 activation by cobalt ions is not known. There are different hypotheses on the role of cobalt in stabilizing the transcription factor HIF-1α, e.g. the replacement hypothesis and the iron oxidation hypothesis.

Taking into consideration results of recent studies by Salnikow and co-workers on the function of the co-factor ascorbic acid in the hydroxylation of prolyl hydroxylases

(Kaczmarek et al., 2009; Salnikow et al., 2004) which support the iron oxidation hypothesis it is proposed to extend the 2nd para ("Cobalt exposure …") by the following wording:

"Recently it has also been shown that HIF-1α stabilization in human lung epithelial cells occurred following exposure to various metal ions including those that cannot substitute for iron in the prolyl hydroxylases (Kaczmarek et al., 2009). The supplementation of cells exposed to metal ions such as cobalt(II) with the reducing agent ascorbic acid abolished HIF-1α protein stabilization."

p.148

Comparison of results of the two 2-year inhalation studies by NTP: cobalt sulphate heptahydrate and cobalt metal

There seems to be a calculation error in the normalization of the exposure concentration of cobalt sulphate to elemental cobalt.

Measurements of the aerosol stoichiometry in the 2-year inhalation carcinogenicity study on cobalt sulphate heptahydrate proved the aerosol delivered to the exposure chamber was primarily cobalt sulphate hexahydrate (NTP 1998; Bucher et al., 1999).

Thus, according to the molecular stoichiometry of cobalt sulphate hexahydrate to cobalt metal the highest actual exposure concentration of 3.0 mg/m³ of cobalt sulphate hexahydrate is equivalent to a concentration of 0.67 mg elemental cobalt per m³. This concentration equates to half of the lowest exposure concentration of cobalt metal (1.25 mg/m³) delivered to the animals in the current study.

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